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# Gamma Interferon Reduces the Synthesis of Fibronectin by Human Keratinocytes



Vera B. Morhenn, M.D.*
Sheila T. Huang, B. A.*
Jonathan N. Mansbridge, Ph.D.**



*Dept. of Dermatology, Stanford University, Stanford, CA USA.
**Psoriasis Research Institute, Stanford, CA USA.

## ABSTRACT

Recombinant gamma interferon (rIFN-γ) has a variety of effects on human keratinocytes including the induction of synthesis and expression of HLA-DR antigen as well as growth inhibition. In order to ascertain whether rIFN-γ affects the keratinocyte's capacity to interact with other skin cells and potentially alter the composition of skin proteins, we tested the effect of rIFN-γ on the secretion of proteins by keratinocytes in vitro.

Keratinocytes grown in serum free medium were treated with increasing concentrations of rIFN-γ (3 U/ml-1000 U/ml). The cells were radiolabeled with 35-S-methionine and the supernatants were harvested, excess 35-S-methionine removed, and the proteins analyzed by polyacrylamide gel electrophoresis. The relative synthesis of several proteins was altered by rIFN-γ treatment. In particular rIFN-γ decreased the synthesis of two proteins, one with a molecular weight of approximately 250 kD and the other 180 kD, and increased the synthesis of an approximately 100 kD protein in a dose dependent manner. Immunoprecipitation with polyclonal antifibronectin antibody showed that the 250 kD protein is the fibronectin monomer. No smaller fragments of fibronectin were immunoprecipitated, suggesting that the reduction in extracellular fibronectin following treatment by rIFN-γ was not a direct result of proteolytic enzyme digestion. Various protease inhibitors were also added to keratinocyte cultures to attempt to reverse the observed protein changes. No significant reversals were noted, further verifying that proteolytic enzyme activity was not responsible for the observed changes in protein patterns.

## INTRODUCTION

Gamma interferon (IFN-γ), a protein secreted by activated T lymphocytes, has pleiotropic effects on human keratinocytes [1]. Recombinant IFN-γ (rIFN-γ) induces the synthesis and expression of HLA-DR antigen at relatively low concentrations [2]. Moreover, rIFN-γ inhibits keratinocyte proliferation and induces the synthesis of intercellular protein(s) [3,4]. The rIFN-γ also induces the expression of intracellular adhesion molecules which may represent the binding site of T cells on IFN-γ exposed keratinocytes [5]. In contrast, rIFN-γ has recently been shown to reduce synthesis of thrombospondin, a wound healing associated with keratinocytes and fibroblast adhesion protein [6]. Since such profound differences in protein synthesis occur in the keratinocyte after rIFN-γ treatment, it seemed plausible that the secretion of certain proteins by these keratinocytes would also be altered. Thus, IFN-γ exposed keratinocytes would not only demonstrate important immunologically relevant alterations limited to the actual site of the cell, but also could affect cells at a distance. In order to explore this possibility, the following experiments were performed.

## MATERIALS AND METHODS

Cell lines. Cultures of human keratinocytes were either purchased from Clonetics Corp., San Diego, CA. or prepared in our laboratory from skin tissue obtained after face lift surgery as previously described [7].

Reagents. rIFN-γ was a generous gift from Genentech Inc., S. San Francisco, CA. G-25-150 Sephadex gel filtration beads were purchased from Sigma Chemical Co., St. Louis, MO.

Protease Inhibitors. Soybean trypsin inhibitor and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St. Louis, MO. Leupeptin and alpha-2-macroglobulin were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN.

Antibodies. Rabbit antifibronectin antiserum was purchased from Transformation Research Inc., Framingham, MA. Goat anti-rabbit IgG was purchased from Tago, Inc., Burlingame CA.

Culture Conditions. Approximately 3 X 10 (4) keratinocytes per dish were plated onto 35 X 10 mm Lux tissue culture dishes (Miles Laboratory, Inc. Naperville, IL) and grown in Keratinocyte Growth Medium (KGM) (Clonetics Corp., San Diego, CA). Cells were incubated in a 5% $CO_2$ incubator at 37°C.

Addition of rIFN-γ and Protease Inhibitors. On day 3, rIFN-γ at 100 U/ml, unless otherwise indicated, was added to each experimental dish. Control dishes received only KGM. Protease inhibitors at the following concentrations: soybean trypsin inhibitor, 100 ug/ml; leupeptin, 300 ug/ml; alpha-2-macroglobulin, 300 µg/ml were added to specific control and experimental plates on day 3. PMSF was added to cultures at a concentration of 1 mmol, however this concentration of PMSF was found to be cytotoxic to cells and therefore use of PMSF was discontinued.

Protein Labeling. On day 5, cultures were incubated with 10 µCi/plate of 35-S-methionine in methionine-free MCDB medium. On day 6, the media were harvested, centrifuged at 1,500 rpm for 8 min. to remove cell debris and filtered through G-25-150 Sephadex gel columns to remove unbound radioactivity. The Sephadex gel had been previously swelled overnight in 10 mM Tris/1mM EDTA pH 8.0 buffer.

Antifibronectin immunoprecipitation. 480 µl of each media sample was mixed with 20 µl undiluted antifibronectin antiserum in a polypropylene tube and incubated at 37 degrees C for 30 min. Binding of antibody to fibronectin was detected by the addition of 500 µl of goat antirabbit IgG, mixing, incubation at 37 degrees C for 30 min., and further incubation at 4 degrees C for approximately 5 days until a precipitate was visible. At this point, samples were centrifuged in a microcentrifuge for 6 min. and the supernatants were separated from the precipitates.

Determination of 35-S-methionine incorporation. Original culture media samples and the supernatants remaining after immunoprecipitation were frozen in a dry ice and ethanol bath, lyophilized overnight, and resuspended in sample buffer. Immunoprecipitates were also resuspended in sample buffer. Samples were pipetted in triplicate onto Whatman filter paper. The filters were stirred in cold 5% TCA for 30 min., rinsed twice in absolute

ethanol, and dried in a warm incubator for 15 min. The filters were placed in scintillation vials, and counted overnight in a beta scintillation counter.

Polyacrylamide Gel Electrophoresis. Proteins were analyzed by discontinuous electrophoresis on 6% - 10% polyacrylamide slab gels in the presence of SDS according to the method of Laemmli [38]. The following high molecular weight protein standards were used: lysosyme (15.100), beta-lactoglobulin (17.850), alpha-chymotrypsinogen (28,250), ovalbumin (41,800), bovine serum albumin (67,450), phosphorylase B (103,150), and myosin (204,900) (Bethesda Research Labs, Gaithersburg, MD). For autoradiography, gels were placed in En$^3$Hance (New England Nuclear) and rotated on a Tek Tator V Rotator (Evanston, IL) at setting 14 for 45 min. followed by a gentle rinse for 30 min. in deionized water. Gels were vacuum dried for 1.5 hrs onto Whatman paper, and exposed to Kodak X-OMAT film for 24 hrs. to 2 weeks depending upon the amount of radioactivity loaded onto the gel. For comparative purposes, equal cpms were loaded onto each gel lane of a series.

## RESULTS

Human keratinocytes were treated with varying concentrations of rIFN-γ ranging from 3 U/ml to 1000 U/ml. Analysis of the secreted extracellular proteins by gel electrophoresis revealed changes in the relative synthesis of several proteins (Fig. 1). Densitometry measurements were performed on the gels, and the absorption values of the 250 kD bands were plotted against the corresponding rIFN-γ concentrations (Fig. 2). At a very low concentration of rIFN-γ (3 U/ml) a small increase in the synthesis of the 250 kD protein was observed (Fig. 2). At higher concentrations, rIFN-γ decreased the synthesis of two proteins, one with a molecular weight of about 250 kD, and the other with a molecular weight of 180 kD (Fig. 1). As shown in Fig. 2, concentrations of 10 U/ml and 30 U/ml rIFN-γ induced a 40% decrease in the 250 kD protein, 100 U/ml and 300 U/ml rIFN-γ induced a 55% decrease, and 1000 U/ml rIFN-γ suppressed the synthesis of the 250 kD protein by greater than 80%.

To further characterize the 250 kD protein, immunoprecipitation with a specific antibody was carried out. Keratinocyte cultures were radiolabeled and half of the cultures were treated with 100 U/ml rIFN-γ. The labeled

medium containing secreted proteins were subjected to immunoprecipitation with rabbit antifibronectin antiserum. The remaining supernatant was saved. The labeled medium, the immunoprecipitate, and the supernatant were analyzed by polyacrylamide gel electrophoresis and autoradiography. The results are shown in Fig. 3. Lane 1 (-rIFN-γ) and lane 4 ( + rIFN-γ) show the total radiolabeled proteins in the medium before immunoprecipitation (Fig. 3a). Following immunoprecipitation, a band at 250 kD and a fainter band at 180 kD were visible in the precipitate (lane 7 (-rIFN-γ) and lane 8 ( + rIFN-γ)) (Fig. 3b). The 250 kD and 180 kD proteins were entirely cleared from the supernatant as shown in lane 13 (- rIFN-γ) and lane 16 ( + rIGN-γ)(Fig. 3c).

Since the addition of rIFN-γ also increased the production of a 100 kD protein (Fig. 1), the possibility arose that the diminished presence of the 250 kD protein might be due to proteolytic cleavage of the this protein resulting in the increased production of lower molecular weight protein fragments. However, no fragments of fibronectin smaller than 180 kD were immunoprecipitated by polyclonal antifibronectin, making this explanation seem unlikely. To further verify that the apparent decrease in fibronectin synthesis was not simply the result of increased protease activity in the culture media of rIFN-γ treated keratinocyte cultures, three protease inhibitors-- soybean trypsin inhibitor, leupeptin, and alpha-2-macroglobulin were added to keratinocyte cultures. Leupeptin inhibits the enzyme proteases plasmin, trypsin, papain, and cathepsin B [9], soybean trypsin inhibitor inhibits trypsin [10], and alpha-2-macroglobulin is considered to be a universal endoproteinase inhibitor [11]. Obtaining a reversal of the changes previously observed for the the 250 kD, 180 kD and 100 kD proteins would suggest that proteolytic cleavage had been responsible for these changes. However, neither treatment by leupeptin (L) (lanes 2,5,8,11,14, and 17) or by soybean trypsin inhibitor (results are not shown) resulted in any reversal of the decrease in 250 kD and 180 kD protein synthesis or the increase in 100 kD observed in control lanes 1,4,7,10,13, and 16. Treatment by alpha-2-macroglobulin did show partial reversal of the 250 kD and 180 kD protein decrease and the 100 kD protein increase (lanes 3,6,9,12,15, and 18) compared to control lanes 1,4,7,10,13, and 16. However, even in the presence of alpha-2-macroglobulin, rIFN-γ treated cells produced less 250 kD and 180 kD proteins and more 100 kD protein than non-rIFN-γ treated cells

(lanes 3,6,9, and 12), suggesting that endoproteinase activity was not the primary cause of the observed changes.

## DISCUSSION

Concentrations of rIFN-γ ranging from 10 U/ml to 1000 U/ml consistently produced inhibition of 250 kD fibronectin and the 180 kD protein synthesis by keratinocytes. In contrast, a very low concentration of rIFN-γ (3 U/ml) appeared to increase the synthesis of these proteins ( Fig. 1, lane 2), suggesting that very low concentrations of rIFN-γ may affect keratinocytes in a manner distinct from higher concentrations. However, it is not clear from these experiments that this increase is biologically meaningful.

Immunoprecipitation of the radiolabeled keratinocyte medium by polyclonal antifibronectin antibody confirmed the 250 kD protein to be the fibronectin monomer. Although we did not test the effect of every protease inhibitor, our evidence thus far suggests that rIFN-γ induces a decrease in the synthesis of fibronectin, and not merely an increased proteolytic breakdown of fibronectin.

The 180 kD protein which was consistently precipitated by the polyclonal antifibronectin antibody is possibly a fragment of fibronectin, the existence of which has been demonstrated by Czop [12]. However, we considered that the 180 kD protein is the glycoprotein thrombospondin, also known as glycoprotein G and thrombin sensitive protein. Thrombospondin, which was originally isolated in activated platelets, was later detected in a variety of tissues including kidney, aorta, embryonic lung, skeletal muscle, and most recently human keratinocytes [13]. Thrombospondin is a 500 kD glycoprotein composed of a trimer of 180 kD subunits. It shares similarities to fibronectin, including a role in adhesion during processes of tissue reorganization such as wound healing [14] and the possession of a RGDS (Arg-Gly-Asp-Ser) peptide in its primary sequence which enable both fibronectin and thrombospondin to bind to GPIIb/IIIa platelet receptor complex [15]. Hence, it is reasonable that thrombospondin shares some of the same epitopes as fibronectin, thus enabling it to cross read with the fibronectin antibody. A weak interaction between thrombospondin and antifibronectin would explain why the 180 kD thrombospondin was precipitated (Fig. 3) and lost during the washing of the precipitate (Fig. 3C).

Confirmation that the 180 kD protein is thrombospondin has recently been reported by Nickoloff et al. [16].

Infected wounds would presumably recruit a large number of activated T-cells with resultant high rIFN-γ levels locally. This lymphokine presumably would decrease fibronectin secretion and thus reduce the amount of fibronectin, an essential protein for the wound healing process. This may explain the clinical observation that the rate of healing of infected wounds is decreased.

The 100 kD protein, the synthesis of which appears to increase during treatment with rIFN-γ, has not yet been identified. We believe that the 100 kD protein is not a breakdown product of fibronectin on the basis that it could not be immunoprecipitated by polyclonal antifibronectin. This protein is likely too small to be a structural protein, and is probably not a breakdown product of thrombospondin since we observed a significant increase in the presence of the 100 kD protein without a corresponding change in 180 kD thrombospondin protein (Fig. 3, lanes 2 and 5).

Alpha-2-macroglobulin has been most extensively characterized as a protease inhibitor. However, recently it was also demonstrated that alpha-2-macroglobulin binds transforming growth factor-beta (TGF-β) in serum. TGF-β, also known as KLIF, has been shown to be secreted by human keratinocytes [16,17] and is capable of stimulating the production of fibronectin [18]. Since alpha-2-macroglobulin results in the entrapment of TGF-β [19], we would expect the addition of alpha-2-macroglobulin to keratinocyte cultures to lead to a decreased synthesis of fibronectin. Interestingly, our results showed the opposite; namely there was an increased production of fibronectin and also of the 180 kD protein in the presence of alpha-2-macroglobulin (Fig. 3, lane 9 (-rIFN-γ), and lane 12 ( + rIFN-γ)). These results were also observed for soybean trypsin inhibitor (results are not shown), but not for leupeptin (Fig. 3, lane 8 (-rIFN-γ), and lane 11 ( + rIFN-γ)). The most likely explanation is that the concentration of the TGF-β generated by keratinocytes under the culture conditions used here is insignificant and that the protease inhibitory effect of alpha-2-macroglobulin outweighs any effect on TGF-β.

## FIGURE LEGENDS

Figure 1. Autoradiograph showing 35-S methionine labeled proteins from keratinocyte medium following the addition of increasing concentrations of rIFN-γ. In particular, treatment of keratinocytes by rIFN-γ appeared to decrease the synthesis of both a 250 kD protein (fibronectin) and a 180 kD protein, and to increase the synthesis of a 100 kD protein in a dose dependent manner. However, these results were reversed at a very low rIFN-γ concentration of 3 U/ml. Lane 1: control. Lane 2: 3 U/ml rIFN-γ. Lane 3: 10 U/ml rIFN-γ. Lane 4: 30 U/ml rIFN-γ. Lane 5: 100 U/ml rIFN-γ. Lane 6: 300 U/ml rIFN-γ. Lane 7: 1000 U/ml rIFN-γ.

Figure 2. Graph showing the decrease $^{35}$S-methionine incorporation into the 250 kD fibronectin band, estimated by densitometry of the autoradiograph. Fifty percent inhibition of fibronectin synthesis occurred at an rIFN-γ concentration of 113 U/ml (shown as a percentage of control) with the addition of increasing concentrations of rIFN-γ (shown as the log function). 10 U/ml and 30 U/ml of rIFN-γ induced a 40% reduction in fibronectin synthesis, 100 U/ml and 300 U/ml of rIFN-γ induced a 55% decrease, and 1000 U/ml of rIFN-γ induced greater than 80% decrease. By contrast, 3 U/ml rIFN-γ appeared to increase fibronectin synthesis.

Figure 3. Immunoprecipitation of 250 kd protein and protease inhibitors on fibronectin synthesis. The removal of 250 kD fibronectin and partial removal of 180 kD protein by immunoprecipitation is shown. Aliquots of media from radiolabeled keratinocyte cultures, either treated or not treated with rIFN-γ, were immunoprecipitated with polyclonal antifibronectin antibody. The labeled medium (A), the precipitate (B), and the remaining supernatants were analyzed by polyacrylamide gel electrophoresis and autoradiography. Lane 1 (-rIFN-γ) and lane 4 ( + rIFN-γ) show the total radiolabeled proteins in the medium before immunoprecipitation. The 180 kd band is very clear while the 250 kd band is faint. Following immunoprecipitation, a clear band at 250 kD and a fainter band at 180 kD were visible in the precipitate - see lane 7 (-rIFN-γ) and lane 10 ( + rIFN-γ). The 250 kD and 180 kD proteins were entirely cleared from the supernatant, as shown in lane 13 (-rIFN-γ) and lane 16 ( + rIFN-γ).

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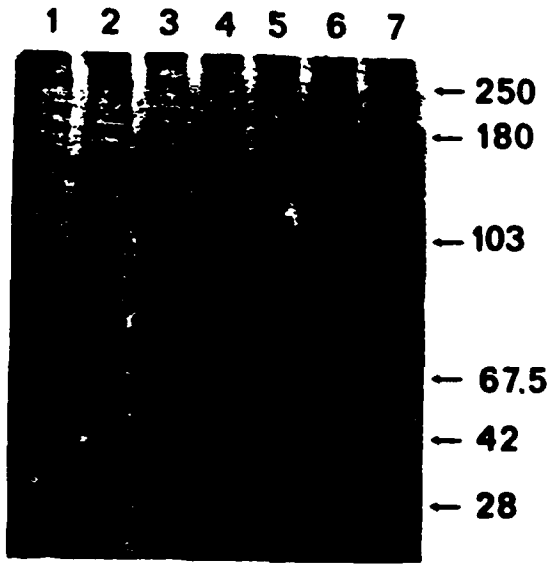
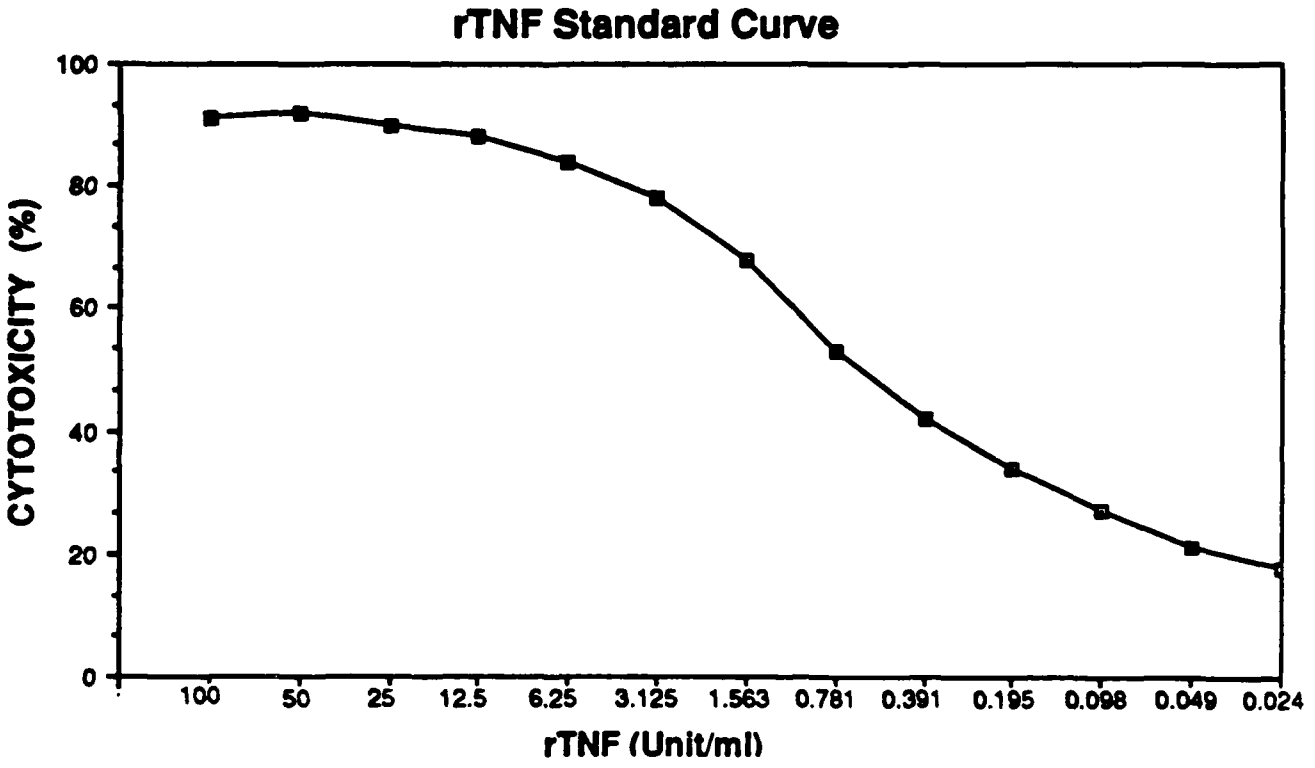



Figure 1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CYTOTOXICITY (%)
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80
60
40
20
0
100
50
25
12.5
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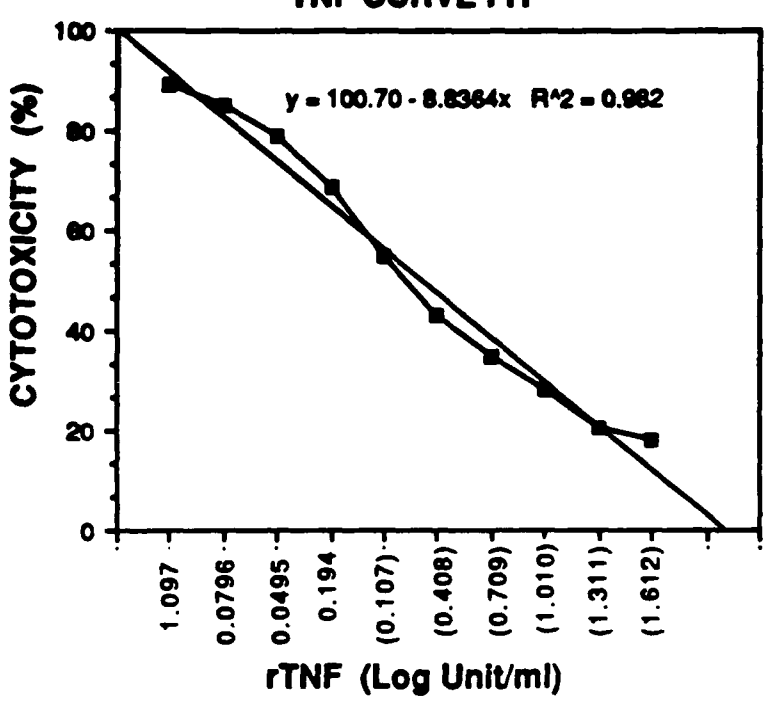
1.563
0.781
0.391
0.195
0.098
0.049
0.024
rTNF (Unit/ml)


TNF CURVE FIT
y = 100.70 - 8.8364x R^2 = 0.982
CYTOTOXICITY (%)
100
80
60
40
20
0
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0.0796
0.0495
0.194
(0.107)
(0.408)
(0.709)
(1.010)
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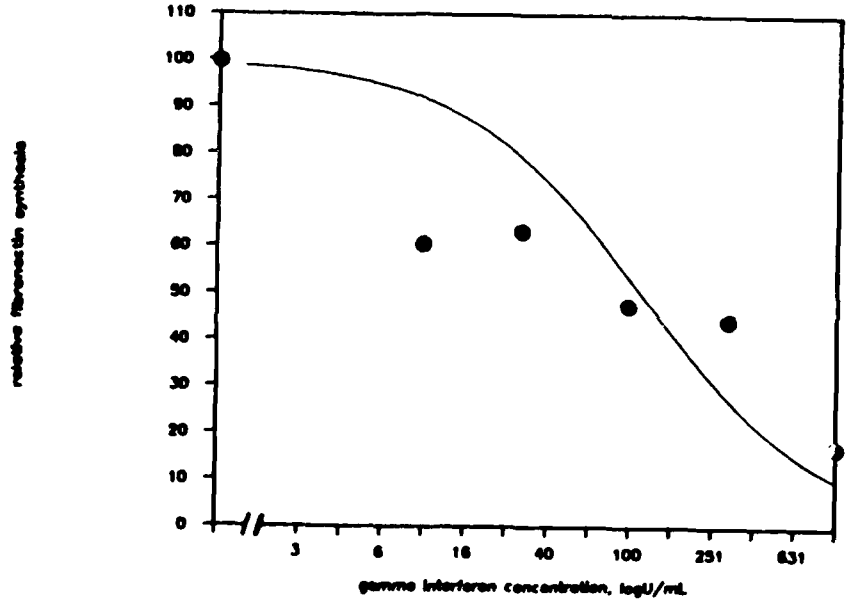
(1.612)
rTNF (Log Unit/ml)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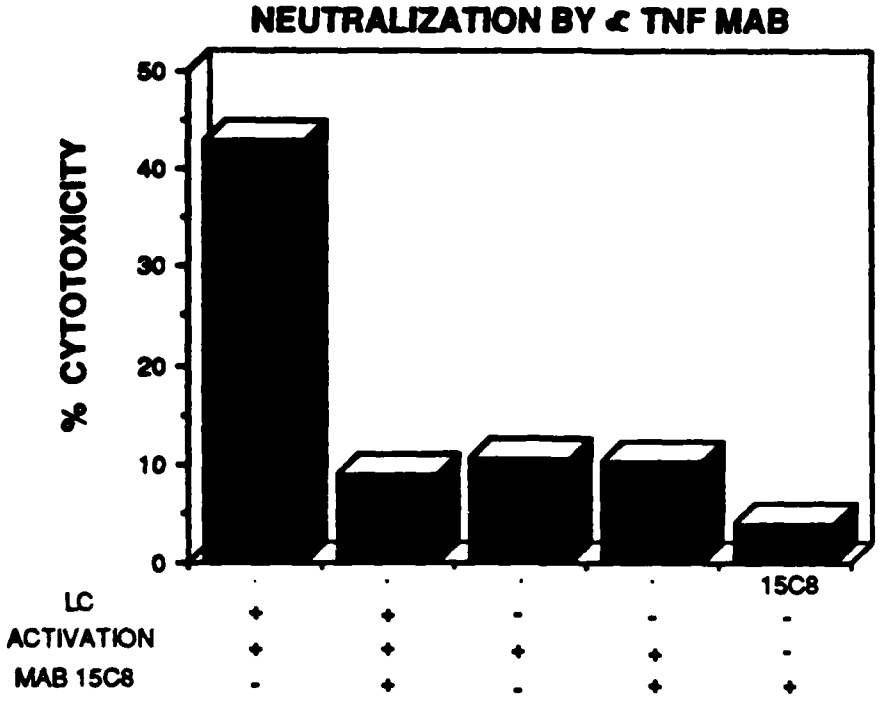
110
100
90
80
70
60
50
40
30
20
10
0
3
6
16
40
100
251
631
gamma interferon concentration, logU/mL

Figure 2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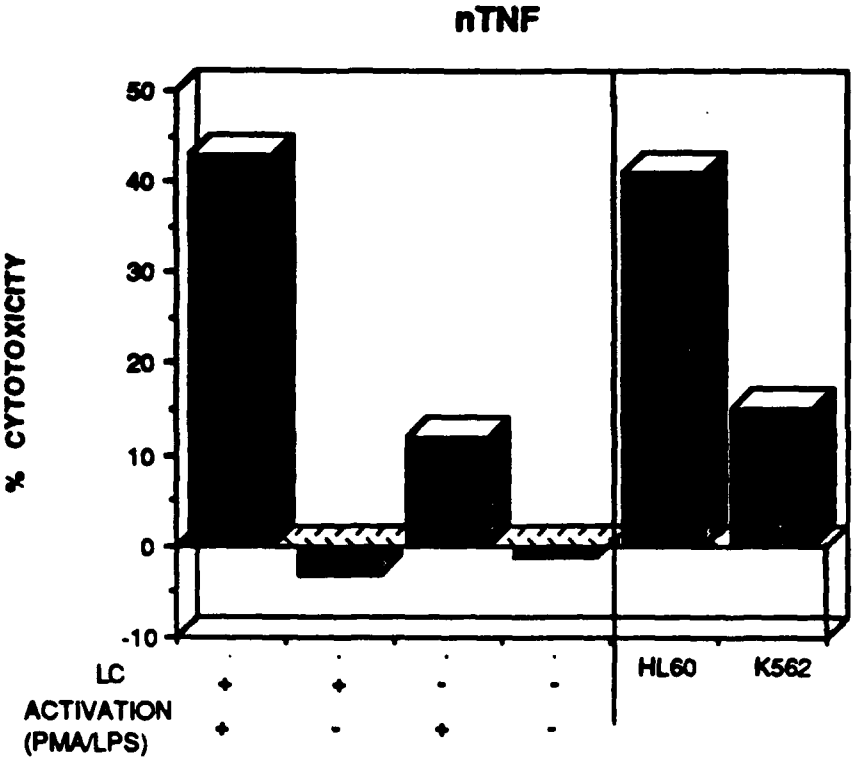
20
10
0
15C8
LC ACTIVATION
MAB 15C8
+ + - - -
+ + + + -
- + - + +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% CYTOTOXICITY
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40
30
20
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ACTIVATION
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+
-
-
+
-
+
-
HL60
K562

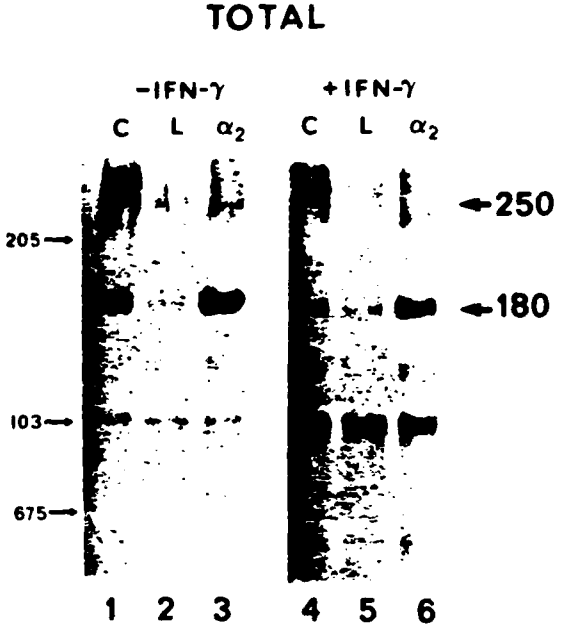
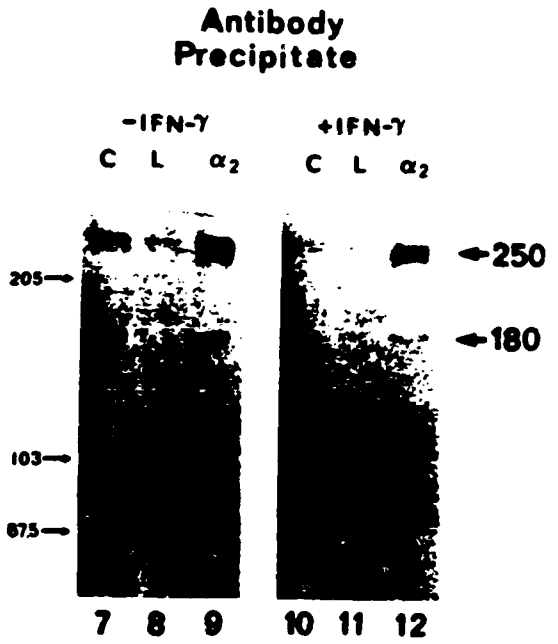



Figure 3-A


Antibody
Precipitate
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+IFN-γ
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C L α2
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103
67.5
250
180
7 8 9
10 11 12

Figure 3-B

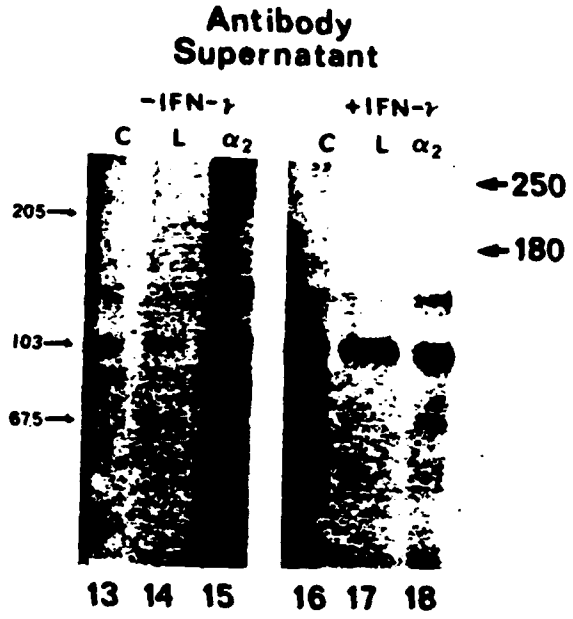
Antibody
Supernatant
-IFN-γ
+IFN-γ
C
L
α2
C
L
α2
205
103
67.5
250
180
13
14
15
16
17
18

Figure 3-C